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Electron Uptake and Delivery Sites on Plastocyanin in Its Reactions with the Photosynthetic Electron Transport System†

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ABSTRACT: French bean plastocyanin is stoichiometrically and specifically labeled upon reduction by $Cr(II)_{aq}$ ions, yielding a substitution-inert Cr(III) adduct at the protein surface. The effect of the modification on the activity of plastocyanin in electron transfer between photosystems II and I has been investigated. The photoreduction and photooxidation by chloroplasts or by photosystem I reaction centers, respectively, of native and Cr(III)-labeled plastocyanin have been compared. It was found that whereas the photoreduction rates of native and Cr-labeled plastocyanin were indistinguishable, the rates of photooxidation of the modified protein were markedly attenuated relative to those of the native one. This difference in reactivity clearly reflects the perturbation of the electron transfer pathway to P_{700}. These findings, in conjunction with the structure of plastocyanin and the locus of Cr(III) binding on its surface, lead to the following interpretation: (a) There are most probably two physiologically significant, electron transfer sites on plastocyanin. (b) The site involved in the electron transfer to P_{700} is most likely in the region of tyrosine-83 and the negatively charged patch proximal to it. By elimination we assume that the second site is centered at the hydrophobic region of histidine-87.

Plastocyanin (Pc)[1] is a "blue" single copper protein functioning as an electron carrier in algae and higher plants. Its site of action in the photosynthetic electron transport chain is commonly believed to be located between cytochrome *f* and P_{700} (Wessels, 1966; Gorman & Levine, 1966; Avron & Shneyour, 1971; Wood & Bendall, 1975; Haehnel et al., 1980;

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[1] Abbreviations: Pc, plastocyanin; RC, photosystem I reaction centers; PS I or II, photosystem I or II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DNP-INT, 2,4-dinitrophenyl ether of 2-iodo-4-nitrothymol; Pc(I) or Pc(II), reduced or oxidized plastocyanin; Cr-Pc, chromium-labeled plastocyanin; cyt *f*, cytochrome *f*; NMR, nuclear magnetic resonance; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

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Bouges-Bocquet, 1977), although a parallel location of Pc and cytochrome *f* between the plastoquinone pool and P_{700} has also been suggested [for review, see Katoh (1977)].

The turnover of Pc in situ has been examined kinetically by flash spectroscopy in broken chloroplasts as well as in algal cells (Haehnel et al., 1980; Olsen et al., 1980; Bouges-Bocquet, 1977). Under continuous illumination, changes in the redox state of endogenous Pc are difficult to follow because of its low concentrations and relatively low extinction coefficient. The photoreduction or photooxidation of externally added Pc can, however, be followed spectroscopically, but the photooxidation requires pretreatment of chloroplasts with a detergent (Katoh & Takamiya, 1963; Nolan & Bishop, 1975).

The three-dimensional structure of Pc has recently been determined and refined for both the oxidized and reduced states, Cu(II) and Cu(I), respectively (Colman et al., 1978; Freeman, 1981). These structures provide a rationale for the unique spectroscopic properties of the copper site. Furthermore, knowledge of the coordination sphere of the metal, its asymmetric location within a hydrophobic core of the molecule, and the proteins surface features all provided the base for dealing with the major problems of the topology and mechanisms of electron transfer. Limiting aspects of the solutions to these problems have emerged in recent years. An outer-sphere electron transfer mechanism is most probably operative, and two areas on the protein surface have been implicated as sites for the reactions. These proposals were based mainly on studies that used inorganic complexes as partners in redox reactions (Lappin et al., 1979) and as probes in NMR spectroscopy (Cookson et al., 1980; Handford et al., 1980). The residues potentially involved in conveying electrons between the copper and the solvent–protein interface were also proposed, primarily by structural conjecture (Colman et al., 1978).

An electron transfer locus on French bean plastocyanin has been identified by employing Cr(II) ions as a reductant (Farver & Pecht, 1981). In this approach, advantage is taken of the chemical properties of both Cr(II) and Cr(III) ions. $Cr(II)_{aq}$, being a strong reductant, provides the affinity to donate electrons to the protein. Further, a very marked difference exists between the ligand exchange dynamics of the ions in these two oxidation states. While $Cr(II)_{aq}$ exchanges its coordinated water molecules at a rate $> 10^8\ s^{-1}$, Cr(III) complexes are highly substitution inert (Basolo & Pearson, 1967).

For French bean plastocyanin, a stoichiometric binding of one Cr(III) per mol of protein was found upon reduction by $Cr(II)_{aq}$ ions. Through proteolytic cleavage a single peptide carrying the Cr(III) was identified (Farver & Pecht, 1981), coinciding with the negatively charged segment proximal to tyrosine-83. As pointed out above, this area has also been implicated in electron transfer reactions with certain outer-sphere reagents (Lappin et al., 1979).

These results raise the question of functional relevance of this electron transfer locus to the physiological reaction between plastocyanin and its partners in the photosynthetic apparatus. Furthermore, having the chromium bound to this area provides the means for examining the involvement of this region in the function of Pc. Therefore, we have compared the photoreactions of native and Cr(III)-labeled plastocyanin with chloroplasts and isolated photosystem I reaction centers.

Experimental Procedures

Materials. Methylviologen and catalase were purchased from Sigma Chemical Co. All other chemicals used were of analytical grade.

Chloroplast Preparation. Broken chloroplasts [class C, according to Hall (1972)] were isolated from pea or lettuce leaves as described by Avron (1961). Chlorophyll concentration was determined according to Arnon (1949).

Reaction centers of photosystem I were isolated and purified from Swiss chard leaves by Dr. N. Nelson as described by Bengis & Nelson (1975) with the diethylaminoethyl (DEAE)-cellulose chromatography as the final purification step.

Plastocyanin Preparations. Plastocyanin was isolated from French bean (*Phaseolus vulgaris*) leaves by a slight modification of the earlier procedure (Milne & Wells, 1970; Farver & Pecht, 1981). The purity of the protein was determined by the A_{278}/A_{597} ratio, which was always less than 1.2. Concentration of native, oxidized Pc was determined from the absorbance at 597 nm, with $\epsilon = 4500\ M^{-1}\ cm^{-1}$ (Milne & Wells, 1970). Native Pc was reduced with H_2 under anaerobic conditions with a catalytic amount of Pt black (Rosen & Pecht, 1976). Cr(III)-labeled Pc(I) was produced as described earlier (Farver & Pecht, 1981). Oxidized, Cr-labeled Pc was obtained by oxidation of Cr(III)-Pc(I) with a stoichiometric amount of $IrCl_6^{2-}$, followed by extensive dialysis. No damage was caused to the protein by this oxidant as judged by both spectroscopic and kinetic properties of the product.

Measurement of Pc Photoreduction or Photooxidation. Photoreduction of exogenously added Pc by chloroplasts and photooxidation by PS I reaction centers (RC) were followed by the decrease or increase (respectively) in the absorption at 590 nm minus that at 540 nm. Measurements were carried out with a DW-2 Aminco dual-wavelength spectrophotometer. All measurements were done at 20 °C with a thermostated cell holder. Actinic illumination at 90° to the measuring beam was provided by a 150-W halogen lamp, filtered through 8 cm of water, and a Schott RG 665 (photoreduction) or RG 715 (photooxidation) filter. The photomultiplier was protected from the actinic light by 1 cm of 50% saturated $CuSO_4$ solution.

Reaction Mixtures. The mixture for Pc photoreduction contained 50 mM KCl, 40 mM NaTricine, pH 8.0, 3 mM NH_4Cl, chloroplasts containing 20 μg of chlorophyll/mL, and 0–6 μM Pc or Cr-Pc as indicated. These conditions ensure that the monitored changes in Pc absorption reflect the rate-limiting step of the reaction (see Results). The reaction mixture for Pc photooxidation contained 50 mM KCl, 40 mM NaTricine, pH 8.0, 20 μM methylviologen, 105 units of catalase/mL, PS I reaction centers containing 14 μg of chlorophyll/mL, and 0–6 μM Pc or Cr-Pc as indicated. In the absence of methylviologen and catalase, the reaction was found to be limited by the electron acceptor rather than electron donor side of PS I.

Data Analysis. Rates of oxidation and reduction of Pc were calculated as pseudo-first-order rate constants. These were obtained by linear least-squares analysis of plots of ln C_t vs. time, where C_t was the concentration of oxidized Pc at time t. As shown below, the reactions of plastocyanin with PS II and RC, respectively, obey the general rate law:

$$\text{rate} = k'[\text{PS II or RC}]C_t \qquad (1)$$

where k'[PS II or RC] is the pseudo-first-order rate constant k_{obsd} for this reaction. k' is a function of total Pc concentration. For the PS I reaction centers preparation used, a molar ratio of 124 chlorophyll/P_{700} was determined by Bengis & Nelson (1975). For chloroplasts, an average value of 500 chlorophylls/cytochrome *f* was used. The nonlinear plots of k' vs. Pc concentration were analyzed according to eq 2 (cf. Results) on an IBM 370/165 computer with a subroutine from the Harwell library (Powell, 1971). The quality of the fit was

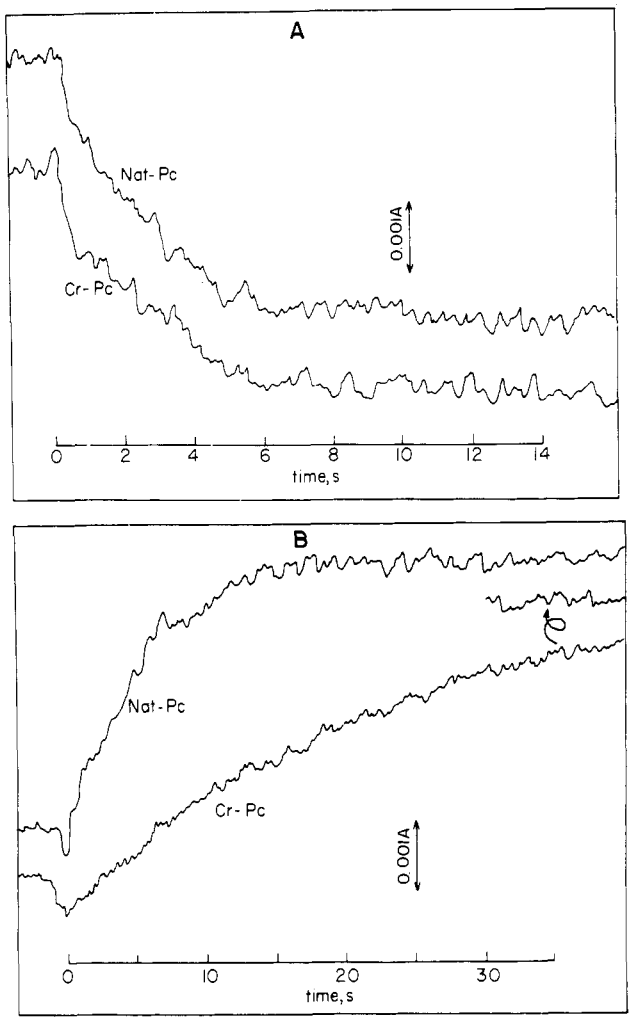

FIGURE 1: (A) Photoreduction of native and Cr-labeled Pc(II) by isolated chloroplasts. Protein concentration was 1.5 μM; chloroplasts contained 20 μg of chlorophyll/mL in 50 mM KCl, 40 mM NaTricine, pH 8.0, and 3 mM NH_4Cl. T = 20 °C. The absorbance was measured at 590 nm minus that at 540 nm. (B) Photooxidation of native and Cr-labeled Pc(I) by PS I reaction centers. Protein concentration was 1.8 μM; PS I reaction centers contained 14 μg of chlorophyll/mL in 50 mM KCl, 40 mM NaTricine, pH 8.0, 20 μM methylviologen, and 105 units of catalase/mL. T = 20 °C. For other experimental details, see Experimental Procedures.

determined from the deviation between the experimental data and the simulated curves.

Results

Photoreactions of Native and Chromium-Labeled Pc. Externally added Pc(II) was photoreduced by broken chloroplasts as illustrated in Figure 1A. Under all the experimental conditions used, both the native protein and the Cr-labeled protein were quantitatively reduced at the same rate. No differences were observed whether pea or lettuce chloroplasts were used. The initial, instant decrease in $A_{590-540}$ seen as the light was turned on was due to a slight leak of actinic light as this decrease was also seen without added Pc. A further slow decrease reflecting Pc(II) reduction was then monitored. The detailed analysis of the kinetics of these reactions is presented under Kinetic Analysis of the Photoreactions. The reaction was measured in the presence of an uncoupler (NH_4Cl) and under saturating light to ensure rate limitation by the added electron acceptor.

Photoreduction of both native Pc(II) and Cr-labeled Pc(II) was found to be sensitive to the PS II electron transfer inhibitors DCMU as well as DNP-INT (data not shown), the latter being an inhibitor of plastoquinone–cyt f electron transfer (Trebst, 1979). This result and the stimulation of cyt f oxidation by externally added Pc (Avron & Shneyour, 1971) suggest that the added Pc(II) reacts at the native site of Pc reaction and not close to the plastoquinone site as proposed by Nolan & Bishop (1975). Still one cannot exclude reduction of Pc by PS I on the basis of these data. It is noteworthy that when DCMU or DNP-INT was added after photoreduction of Pc had been completed, no photooxidation by PS I could be detected. Swollen chloroplasts (prepared in 10 mM NaCl), which are more permeable, yielded the same results (not shown).

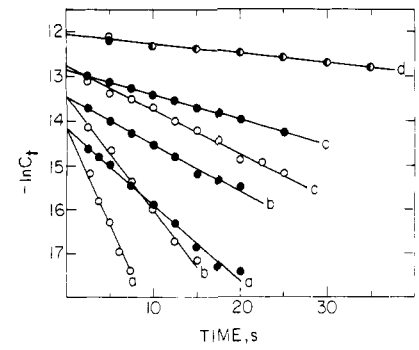

FIGURE 2: Time course of photooxidation of native and Cr-labeled Pc(I) with PS I reaction centers at varying total concentrations of Pc. C_t is the concentration of Pc(I) at time t. The total Pc concentrations were (a) 0.73, (b) 1.45, (c) 2.90, and (d) 5.80 μM. (○) Native Pc; (●) Cr-labeled Pc. Other experimental conditions are as in Figure 1B.

Photooxidation of native and Cr-labeled Pc(I) by isolated PS I reaction centers could easily be measured, and Figure 1B shows typical traces of the reaction. Upon illumination, a fast decrease in $A_{590-540}$ due to P_{700} oxidation was followed by a slower increase in absorption, which reflects Pc(I) oxidation. The former but not the latter was observed in the absence of Pc. The experiments were done in the presence of methylviologen and catalase in order to avoid rate limitation by the electron acceptor and perturbations by produced peroxide, respectively. Photooxidation of the Cr-labeled Pc differed kinetically from that of the native one.

Kinetic Analysis of the Photoreactions. The rates k_{obsd} of photoreduction and photooxidation of both native Pc and Cr-Pc were obtained from the slopes of plots of ln C_t vs. time at Pc concentrations in the range from 0.4 to 5.8 μM. k_{obsd} was found to be proportional to the concentrations of chloroplasts or PS I reaction centers, respectively, indicating first-order kinetics in each of these species. $k_{obsd} = k'$[PS II or RC], where k' was found to be a function of total Pc concentration. Figure 2 shows the time course of photooxidation of reduced native and Cr-labeled Pc by isolated PS I reaction centers. Two important features can be noted: (1) a large difference in reactivity between the native protein and the Cr-labeled protein at the lower Pc concentrations and (2) a steady decrease in rate with increasing total Pc concentration. This is more directly demonstrated in Figure 3A, which shows a plot of k' against $[Pc]_{total}$.

The photoreduction of oxidized Pc by chloroplasts was also found to exhibit an inverse relationship between rate and total Pc concentration (cf. Figure 3B). However, in this case, there is no difference between the reactivity of the native and Cr-labeled protein.

Plots of $1/k'$ against $1/[Pc]_{total}$ concentration are in all cases nonlinear. The expression found to fit best that behavior contains an inverse square term in total Pc concentration.

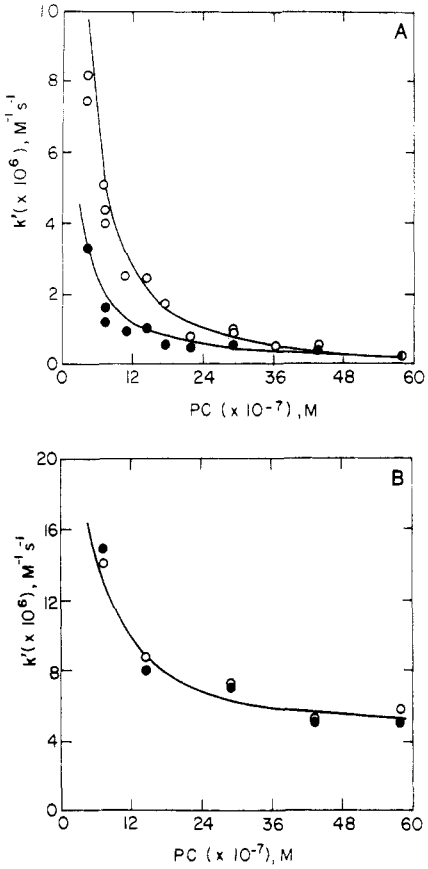

FIGURE 3: (A) Dependence of k' on Pc for photooxidation of native (○) and Cr-labeled (●) Pc(I). Assay conditions are as given in legend to Figure 1B. (B) Dependence of k' on Pc concentration for photoreduction of native (○) and Cr-labeled (●) Pc(II). Assay conditions are as given in legend to Figure 1A.

Table I: Values of the Kinetic and Thermodynamic Constants As Defined by Equation 1

reaction	A (M^{-1} s^{-1})	B (M^{-1} s^{-1})	C (M^{-1})	D (M^{-1})
native Pc(II) + chloroplasts	2.3×10^{10}	4.0×10^{6}	3.3×10^{9}	28
Cr-Pc(II) + chloroplasts	2.3×10^{10}	4.0×10^{6}	3.3×10^{9}	28
native Pc(I) + RC	1.8×10^{8}	0.3	3.0×10^{7}	6.7×10^{5}
Cr-Pc(I) + RC	6.9×10^{7}	2.0	4.7×10^{7}	5.5×10^{4}

Results from up to 14 different experiments were found to excellently fit eq 2:

$$k' = \frac{A + BC[\mathrm{Pc}]_{\mathrm{total}}}{1 + C[\mathrm{Pc}]_{\mathrm{total}} + CD[\mathrm{Pc}]^2_{\mathrm{total}}} \quad (2)$$

Here k' is the calculated second-order rate constant for Pc oxidation or reduction, $[\mathrm{Pc}]_{\mathrm{total}}$ is the total Pc concentration, and A, B, C, and D are constants. A mechanistic consideration of this expression is given under Discussion. The results of the nonlinear fitting are summarized in Table I.

Discussion

Kinetics of Native Pc. The time course of the redox process of Pc with the respective photosynthetic components was found to be complex. The values of k' decrease with increasing total Pc concentration for both the oxidation (Figure 3A) and reduction (Figure 3B) processes. This rate dependence on the Pc concentration shows that the rate-determining step in the electron transfer process is indeed the one in which Pc is involved: most probably electron transfer from cyt f to Pc(II) in the chloroplast reaction and electron transfer from Pc(I) to P_{700} in the reaction with isolated PS I reaction centers.

A number of mechanisms can be devised to describe the observed kinetic behavior. One possibility is that under the examined in vitro conditions, abortive complexes can be formed between the reactants, with a geometry that prevents interaction with the electron transfer sites on Pc. Such a "dead-end" complex mechanism had been proposed earlier for the oxidation of cytochrome c by mitochondrial cytochrome c oxidase, a reaction that exhibits a remarkable kinetic similarity to the one reported here (Errede & Kamen, 1978). The simplest expression for a mechanism involving a dead-end complex formation would lead to a rate law of the form

$$k' = \frac{AB[\mathrm{Pc}]_{\mathrm{total}}}{1 + B[\mathrm{Pc}]_{\mathrm{total}}} \quad (3)$$

However, this expression does not fit our experimental observations. In particular, the plots of $1/k'$ vs. $[\mathrm{Pc}]_{\mathrm{total}}^{-1}$ are nonlinear. Instead it was found that a mechanism presented by Errede & Kamen (1978, Table IV) leading to the expression given in eq 2 gave an excellent fit. The uniqueness of this mechanism cannot be demonstrated by kinetic measurements. Alternative interpretations of the data in terms of a single reaction site on Pc affected to different extents in its reactivity toward P_{700} and cyt f by the Cr(III) label cannot be excluded. Still, it serves as a convenient basis for discussing differences in the reactivity of Pc between the native and Cr-labeled protein and is fully consistent with the available structural information on Pc. The mechanism is consistent with all the experimental data obtained for Pc in both its reaction with chloroplasts and with PS I reaction centers. The calculated constants given in Table I would then have the following meaning: In the Pc(II) reduction by cyt f, A represents the rate constant for the electron transfer from chloroplasts (cyt f) to Pc(II) (2.3×10^{10} M^{-1} s^{-1}), and B is the rate constant of electron transfer from a dead-end Pc–cyt f complex to Pc(II) (4.0×10^{6} M^{-1} s^{-1}). The exceptionally high value of A is larger than the theoretical diffusion-controlled rate of macromolecules in solution. This most probably reflects the complex nature of the process involving membrane-bound reactants and possibly two-dimensional diffusion in its plane. C and D represent the stability constants of dead-end complexes between membrane-bound cyt f and one or two molecules of Pc (3.3×10^{9} and 28 M^{-1}, respectively). The latter number indicates that complex formation between membrane-bound cyt f and two Pc molecules is negligible.

In the electron transfer reaction between Pc(I) and isolated PS I reaction centers, A represents the rate constant for electron transfer from Pc(I) to P_{700} (1.8×10^{8} M^{-1} s^{-1}), and B is now the very slow rate of electron transfer from Pc(I) to a reaction center bound to another Pc in a dead-end complex. C and D have the same meaning as before, and it is noteworthy that complexes of RC with one and with two molecules of Pc both seem to exist (Table I), although they are abortive. Wood (1974) has reported a pseudo-first-order cyt f oxidation by excess Pc in solution (both isolated from parsley) with a rate constant of 3.6×10^{7} M^{-1} s^{-1} at 25 °C, pH 7.0. For the photoreduction of exogenously added Pc by broken chloroplasts, Wood & Bendall (1975) also reported a pseudo-first-order rate dependence, with $k = 2 \times 10^{6}$ M^{-1} s^{-1} [25 °C; pH 7.0; with 4 μM Pc(II) and 1.04 μg of chlorophyll/mL]. For Pc(I) oxidation by PS I (in digitonin-treated chloroplasts), they reported of a pseudo-first-order rate constant of 8×10^{7} M^{-1}

s^{-1} (with 2.5 μM Pc and 0.41 μg of chlorophyll/mL). However, no rate dependence on Pc concentration was reported in the above study, and it is therefore difficult to compare it directly with the present kinetic data. Using the parameters given in Table I for Pc(II)–cyt *f* in chloroplasts and the Pc concentration employed in the work of Wood & Bendall (1975), we calculated (eq 2) $k = 5.7 \times 10^6$ M^{-1} s^{-1} for the photoreduction. This value is in good agreement with the one reported above, considering the different experimental conditions.

Kinetics of Cr-Labeled Pc. Turning now to the electron transfer reactions involving Cr-labeled Pc, we also observed the inverse relation between k' and total Pc concentration (Figure 3). This means that all the data for the Cr-labeled Pc reactions can be discussed in terms of the same mechanism as that proposed for the native protein. What is obvious already from Figures 1B and 2 is that at low Pc concentrations, electron transfer from the labeled plastocyanin to P_{700} is significantly slower than for native Pc. This effect is also clearly illustrated in Figure 3A, as well as in the results of the nonlinear fitting given in Table I. The rate constant A for electron transfer between Pc(I) and a reaction center is slowed down by almost a factor of 3 when Pc is labeled with Cr(III). This is in contrast with the observed insensitivity of the photoreduction of Pc(II) by chloroplasts to the Cr(III) presence. These observations make a further discussion of the sites of interaction between Pc and its physiological partners possible.

Electron Transfer Pathways in Pc. The specific binding site of the Cr(III) ion on the surface of Pc has been identified as a highly conserved sequence of four acidic residues: Asp-42, Glu-43, Asp-44, and Glu-45 (Farver & Pecht, 1981). This binding site is very close to Tyr-83 as seen in the three-dimensional model of the native protein (Colman et al., 1978) and implied by fluorescence studies of Cr-labeled Pc (Farver & Pecht, 1981). The distance between the copper center and the proposed binding site for Cr(III) is ~12 Å, and the intervening region contains an array of highly invariant aromatic residues (Colman et al., 1978). These aromatic residues may play a role in the electron transfer between the copper ion and the protein–solvent interface by $d\pi$–π^* delocalization.

It is of interest to note that two independent sites of interaction for outer-sphere electron transfer agents (Lappin et al., 1979) or their redox inert analogues (Cookson et al., 1980; Handford et al., 1980) have been identified on Pc. Positively charged reagents were found to interact with Tyr-83, which is in the area where Cr(III) is bound after the inner-sphere reduction of Pc(II) by Cr(II). In contrast, negatively charged outer-sphere redox agents were found to interact with a completely different site near the exposed imidazole ring of the copper-ligating His-87 on Pc.

The finding that the photoreduction of Pc(II) by chloroplasts is not affected by the presence of the bound Cr(III) whereas photooxidation of Pc(I) by P_{700} is markedly attenuated by this modification now suggests two interesting ideas: (a) the area of Cr(III) coordination near Tyr-83 is involved in the reduction of P_{700}; (b) in accordance with the above observation for inorganic outer-sphere reagents, there are at least two physiologically significant sites for electron transfer on Pc. Examination of the three-dimensional structure of Pc has led to the suggestion of the existence of more than one reactive site (Colman et al., 1978), and this study lends this its first experimental support. Furthermore, the findings reported here may provide evidence for directionality in the physiological Pc reactivity, i.e., distinct sites operative in electron uptake and delivery.

It is important to note that the presence of Cr(III) only attenuates the electron transport rate from the Cu redox center to P_{700}. The area on Pc to which Cr(III) is bound is highly negatively charged due to several neighboring carboxylates (Colman et al., 1978; Freeman, 1981). Thus, the attached Cr(III) ion probably perturbs the recognition site on Pc for the PS I reaction center.

Another potential electron pathway proposed for Pc is via the imidazole of His-87, which is one of the Cu ligands described above. The structural analysis has shown that an edge of the ring is exposed to the solvent and that it is surrounded by a large number of highly conserved hydrophobic amino acid residues (Colman et al., 1978). On the basis of the above kinetic results, which show that the photoreduction of Pc(II) by chloroplasts is independent of the Cr(III) label, we propose that the electron transfer between cytochrome *f* and plastocyanin proceeds via the His-87 pathway.

Interaction of Pc with Its Electron Donor and Acceptor within the Thylakoid Membrane. The respective assignments of the hydrophobic interaction of Pc with its membranal electron donor (cyt *f*) and the anionic hydrophilic patch with the electron acceptor (P_{700}) are fully compatible with two recent reports in which rather different approaches were employed. Haehnel et al. (1980) have shown that $MgCl_2$ and osmotic strength affected flash-induced fast kinetics of P_{700} reduction but not cytochrome *f* oxidation in broken chloroplasts. Davis et al. (1980) have reported that Mg^{2+} increased the affinity of PS I particles toward eukaryotic Pc(I) but that it lowered the affinity for prokaryotic Pc. Plastocyanin from these two sources differs in that the second lacks the negatively charged patch of the former. These results lend further support to the proposed hydrophilic nature of Pc–PS I interaction.

Cytochrome *f* is thought to be embedded within the thylakoid membrane since drastic methods are required for its isolation from the membrane (Wood, 1974). This argues well for the proposed hydrophobic interaction with Pc. The orientation of the latter in the thylakoid membrane is, nevertheless, still a matter of controversy (Hauska et al., 1971; Schmidt et al., 1975; Böhme, 1978).

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Coupling Factor for Photophosphorylation Labeled with Eosin Isothiocyanate: Activity, Size, and Shape in Solution†

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ABSTRACT: The coupling factor for photophosphorylation (CF1) was covalently labeled with eosin isothiocyanate (ESCN). We found extra binding sites for the label when a protonmotive force existed across the thylakoid membrane during incubation with ESCN. Two out of three such extra sites are located on the γ subunit of CF1. As judged from the (oxygen-dependent) triplet lifetime of bound ESCN, these extra sites are more deeply buried within CF1 than those sites which are already accessible to ESCN in the absence of a protonmotive force. Labeling of the extra sites by only one ESCN per CF1 greatly reduced the activities of the enzyme (more strongly ATP synthesis and Mg^{2+}-dependent hydrolysis of the membrane-bound CF1 than the Ca^{2+}-dependent hydrolysis). On the other hand, a load of up to five ESCN's when bound in the absence of a protonmotive force had hardly any effect. We isolated and purified active CF1 which was labeled in the absence of a protonmotive force. We studied the rotational diffusion of the isolated enzyme by a photoselection technique aiming at the triplet state of bound eosin. A biphasic decay of the photoinduced linear dichroism of the absorption changes of eosin was observed. The more rapid component was only slightly dependent on the solvent viscosity and therefore attributed to librational motion of the label within the protein. The decay of the slower component was linearly related to the solvent viscosity and therefore attributed to protein rotational diffusion. The theoretical evaluation of the data led us to conclude that isolated CF1 is ellipsoidal rather than spherical in shape, with an axial ratio which is greater than 2.1 if it is prolate and smaller than 1/2.6 if it is oblate.

Synthesis of ATP in green plants is mediated by the coupling factor (CF1).[1] This enzyme is composed from five types of subunits, and it is bound to the thylakoid membrane via its counterpart (CF0), which acts as a proton well [for recent reviews, see Nelson (1976), Kagawa et al. (1979), and Shavit (1980)]. It seems generally accepted that these two together act as a proton-translocating ATP synthase as proposed by Mitchell (1966). In comparison with the ATP synthase complexes of bacteria and of mitochondria, the one of green plants is distinguished by its apparent irreversibility in the absence of a protonmotive force (Petrack & Lipman, 1961; Kaplan et al., 1967; Mills & Hind, 1979). It is activated under conditions which are also known to induce large conformational changes of CF1 when a protonmotive force is generated by illumination, by an acid-base jump, or by externally applied electric field pulses (Ryrie & Jagendorf, 1971; McCarty & Fagan, 1973; Graeber et al., 1977; Weiss & McCarty, 1977; Kraayenhof & Slater, 1975). The same conditions promote the release (or the exchange) of tightly bound nucleotides

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[1] Abbreviations: CF1, coupling factor for photophosphorylation; ESCN, eosin isothiocyanate; 5-IAF, 5-(iodoacetamido)fluorescein; DTT, dithiothreitol; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; fwhm, full width at half-maximum.

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